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Wisteria floribunda gall extract inhibits cell migration in mouse B16F1 melanoma cells by regulating CD44 expression and GTP-RhoA activity

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Abstract

Extracts from galls grown on *Wisteria floribunda* are used as an anti-tumoral preparation in oriental traditional medicine. Here, we investigated the molecular mechanism of this anti-tumoral effect by first examining whether the extract inhibited cell migration in a B16 cell-based wound healing assay. The gall extract delayed wound healing in a dose- and time-dependent manner, indicating that one or more components of the fraction inhibited cell migration. Examination of two molecules known to be involved in metastasis, CD44, and RhoA-GTP, revealed that the gall extract decreased CD44 expression in a concentration-dependent manner, and also increased RhoA-GTP activity in comparison to untreated controls. Taken together, these results suggest that the *Wisteria* gall extract may inhibit cancer cell migration via inhibition of CD44 mRNA expression and activation of the GTP-RhoA protein.

Keywords: *Wisteria floribunda*; Fabaceae; Cell migration; Melanoma cells; RhoA; CD44

1. Introduction

Wisteria floribunda (Willd.) DC, a member of the *Fabaceae* family, is widely grown in East Asian countries, including Korea, where it produces violet-colored flowers in early summer (Yoon, 1992). The plant occasionally produces galls on the stem. Although the exact mechanisms are unknown, gall formation is thought to be triggered by deposition of moth eggs. These possible causes suggest that the gall is associated with the *Wisteria* plant defense mechanisms, which may involve intra-gall production of toxins (Yoon, 1992). Interestingly, many Oriental medicinists use *Wisteria* gall extracts for treating breast cancer, stomach cancer, or rheumatoid arthritis patients, suggesting that these toxins may have wide-ranging uses.

Here, we are the first to systematically test possible molecular mechanisms for the anti-tumoral effects of the *Wisteria* gall extract by examining its action in wound healing and its effect on two tumor-related molecules. Many cell adhesion molecules are involved in cancer metastasis, including the integrin receptors, cadherins, immunoglobulins, ICAM, and CD44. Of these, CD44 is a membrane-bound protein that participates in cell motility, proliferation, and cell–cell interactions in normal and cancer cells (Turley, 1984; Herrera-Gayol and Jothy, 1999). Furthermore, the small GTPases, such as RhoA, Rac, and Cdc42, are members of the signaling on actin cytoskeleton homeostasis via actin polymerization and depolymerization, and membrane changes (Engers et al., 2001; Shah et al., 2001). As cancer metastasis and proliferation may be modulated via the cell motility and adhesion

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properties controlled by CD44 and RhoA, we postulated that these molecules might be modulated by *Wisteria* gall extract.

To examine this, we treated B16 cells with *Wisteria* gall extract and examined the effect on wound healing (an assay of cell migration) and expression of CD44 and RhoA. We found that treatment with the *Wisteria* gall extract was associated with inhibition of wound healing, decreased CD44 mRNA expression, and activation of GTP-RhoA in B16 cells, suggesting that this extract should be studied further as a possible new anti-cancer agent.

2. Material and methods

2.1. Cells, reagents, and extract preparation

B16-F1 (B16) cells (a mouse melanoma cell line) were obtained from the American Type Culture Collection (ATCC No. CRL-6323, Manassas, VA) and cultured in RPMI containing 10% FBS. The gall on *Wisteria floribunda* (Willd.) DC was collected from a senior staff of Kyungpook National University, Daegu, Korea (between July and September 2003) near GumJung Mt. and identified by the staff of the Biology Department as tree and gall on *Wisteria floribunda* (Willd.) DC (family: *Fabaceae*). Voucher specimens of the plant and gall have been deposited in the University's Herbarium (voucher specimen #2003-256). Gall lumps were carefully collected from *Wisteria floribunda* (Fig. 1B) and extracted with chloroform. In brief, gall produced on *Wisteria floribunda* was grinded (50 g) and extracted with three volumes of methanol overnight. The methanolic fraction (water:methanol = 9:1, v/v) was further re-extracted with chloroform. The chloroform fraction was taken to dryness (0.5 g) and suspended with dimethylsulfoxide as an active mixture (100 mg/ml).

2.2. RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR)

Total cellular RNA was extracted from B16 cells using the TRIzol reagent (Gibco BRL, Grand Island, NY). First strand cDNA was synthesized from 1 μg of total RNA using Oligo(dT) reverse transcriptase (iNtRON Biotechnology, Sungnam, Korea), and PCR amplified with specific primers for mouse CD44 (forward, 5′-TCG ATT AGA ATG TAA CCT GCC-3′; reverse, 5′-TGG TGT GTT CTA TAC TCG CCC-3′) and GAPDH (forward, 5′-ATG TTC CAG TAT GAC TCC AC-3′; reverse, 5′-GCC AAA GTT GTC ATG GAT GA-3′) as an internal control. The resulting PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining.

2.3. Wound healing assay

An in vitro wound healing assay was used to assess cell migration in B16 cells treated with various concentrations of the *Wisteria* gall extracts. Linear wound tracks were delineated by thin tape strips affixed to the well bottoms of six-well plates (Greiner, Longwood, FL) and cells were plated at 1×10^7 cells/well. The plates were then cultured in a CO2 incubator for 5 h at 37 °C, to allow cells to attach. The tape lines were then removed to create 'wounds' in the cell layer and the remaining cells were treated with various concentrations of the *Wisteria* gall extract. Photographs were taken at 0, 16, 24, and 40 h and used to compare the inhibitory effects.

2.4. Activities of RhoA

Cellular amounts of the GTP-bound form of RhoA were tested for *Wisteria* gall-induced activation using an Activation Kit (Pierce, Rockford, IL) according to the manufacturer's instructions. In brief, cells (1×10^6 cells) were centrifuged at 16,000 × *g* and the supernatant (cell lysate) was incubated for 1 h at 4 °C with GST-Rhotekin-RBD (25 μl/lane) loaded on a SwellGel immobilized Glutathione Disk (Pierce, Rockford, IL). The Glutathione Disk-bound proteins were solubilized with 2 × SDS (125 mM Tris–HCl, pH 6.8, 2% glycerol, 4% SDS (w/v), 0.05% mercaptoethanol, and 0.05% bromophenol blue). Isolated RhoA-GTP protein from the disk was separated out by 12% SDS-PAGE and

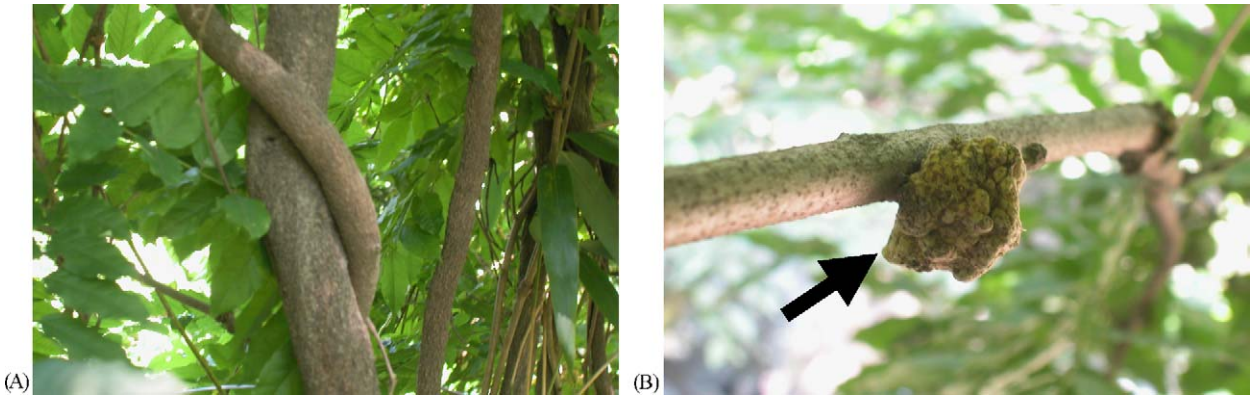

Fig. 1. *Wisteria floribunda* (A) and its gall (B). In (B) the arrow indicates the gall.